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Your Reference: JBV/PKA/P31540

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15 JUL 1996

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Request for grant of a Patent

Form 1/77 **Patents Act 1977**

❶ Title of invention

1 Please give the title of the invention COMPOUNDS

❷ Applicant's details

❑ **First or only applicant**

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4. Agent's or applicant's reference number *(if applicable)*

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5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

❻ Declaration of priority

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❼ Inventorship

7. Are you (the applicant or applicants) the sole inventor or the joint inventors?

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❽ *Please supply duplicates of claim(s), abstract, description and drawings).*

❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77			
Claim(s)		Description	49
Abstract		Drawing(s)	

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)*	
Translation(s) of Priority documents *(please state how many)*	
Patents Form 7/77 - Statement of Inventorship and Right to Grant	
Patents Form 9/77 - Preliminary Examination Report	
Patents Form 10/77 - Request for Substantive Examination	

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❾ Request

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COMPOUNDS

This invention relates to polypeptide derivatives, their use in therapy and methods and intermediates for their production.

Essentially all protein drugs are administered as solutions and function *in vivo* in the solution phase. In biochemistry and pharmacology, however, a large number of control and mediator proteins are associated with or function within the plasma membranes of cells. Except for soluble, truncated versions of one class of these molecules, no membrane-associated proteins have been developed as therapeutic agents. There are two main reasons for this situation. Firstly, overexpression of proteins that are retained in the membranes of the producer cells is limited by the the low capacity of membranes for proteins and often by the toxic effects of retention when expression is intrinsically efficient. Secondly, extraction of these proteins from membranes requires detergents or organic solvents, often results in inactivation of the protein, leads to difficulties in achieving the high purity needed for drug use and usually gives a product which is hard to formulate for intravenous administration.

Soluble, truncated versions of membrane-associated proteins overcome the production difficulties associated with full length proteins. However such truncated molecules lack the membrane binding capability and specificity of the full length proteins which properties may be advantageous or even essential to the desired therapeutic activity.

The main classes of interaction of proteins with membranes can be summarised as follows:

1. Direct and specific interactions with phospholipid head groups or with other hydrophilic regions of complex lipids or indirectly with proteins already inserted in the membrane. The latter may include all the types of intrinsic membrane protein noted below and such interactions are usually with extracellular domains or sequence loops of the membrane proteins:

2. Through anchoring by a single hydrophobic transmembrane helical region near the terminus of the protein. These regions commonly present a hydrophobic face around the entire circumference of the helix cylinder and transfer of this structure to the hydrophilic environment of bulk water is energetically unfavourable.

3. Further anchoring is often provided by a short sequence of generally cationic aminoacids at the cytoplasmic side of the membrane, C-terminal to the transmembrane helix:

4. Through the use of multiple (usually 2-12, commonly 4-7 and 10-

amphipathic behaviour - an outer hydrophobic face and an inner more hydrophilic one being identifiable within a helix bundle located in the lipid bilayer;

5. Through postranslationally linked phosphatidyl inositol moeities (GPI-anchors). These are generated by a specific biosynthetic pathway which recognises and removes a specific stretch of C-terminal aminoacids and creates a membrane-associating diacyl glycerol unit linked *via* a hydrophilic carbohydrate spacer to the polypeptide;

6. In a related process, single fatty acid groups such as myristoyl, palmitoyl or prenyl may be attached postranslationally to one or more sites in a protein (usually at N- or C-termini). Again, amino acids (such as the C-terminal CAAX box in *Ras* proteins) may be removed.

The present invention provides a soluble derivative of a soluble polypeptide, said derivative comprising two or more heterologous membrane binding elements with low membrane affinity covalently associated with the polypeptide which elements are capable of interacting independently and with thermodynamic additivity with components of cellular membranes exposed to extracellular fluids.

By 'heterologous' is meant that the elements are not found in the native full length protein from which a soluble protein may be derived.

By 'soluble polypeptide' is meant a truncated derivative of a full length protein which lacks its natural membrane binding capability, and/or a polypeptide which has a solubility level in aqueous media of > 100µg/ml.

By 'membrane binding element with low membrane affinity' is meant that the element has significant affinity for membranes, that is a dissociation constant greater than1µM, preferably 1µM-1mM. The elements preferably have a size <5kDa.

The derivative should incorporate sufficient elements with low affinities for membrane components to result in a derivative with a high (preferably 0.01 - 10nM dissociation constant) affinity for specific membranes. The elements combine so as to create an overall high affinity for the particular target membrane but the combination lacks such high affinity for other proteins for which single elements may be (low-affinity) ligands.

The elements should be chosen so as to retain useful solubility in pharmaceutial formulation media, preferably >100µg/ml. Preferably at least one element is hydrophilic.

The invention thus promotes localisation of a therapeutic protein at cellular membranes and thereby provides one or more of several biologically significant effects with potential therapeutic advantages including:

Potency: If the protein is a receptor and an agonist or antagonist activity is localised on the same surface as the receptor itself, an increase in effective concentration may result from the reduction in the diffusional degrees of freedom.

Pharmacokinetics and dosing frequency: Interaction of a derivatised protein with long-lived cell types or serum proteins would be expected to prolong the plasma residence time of the protein and produce a depot effect through deposition on cell surfaces.

Specificity: Many clinically important pathological processes are associated with specific cell types and tissues (for example the vascular endothelium and the recruitment thereto of neutrophils bearing the sialyl Lewis ^{x} antigen to ELAM-1, see below). Hence targeting the modified protein to regions of membrane containing pathology-associated membrane markers may improve the therapeutic ratio of the protein targeted.

It will be appreciated that all associations of heterologous amino acid sequences with a polypeptide which is a soluble derivative of a human protein will need to be assessed for potential immunogenicity, particularly where the amino acid sequence is not derived from a human protein. The problem can be minimised by using sequences as close as possible to known human ones and through computation of secondary structure and antigenicity indices.

Examples of protein therapeutic agents which may be modified according to the processes of the invention include but are not restricted to the following:

Base Protein	**Cell Target**	**Therapeutic Application**
IL-4 Y124D mutein	B-cells	Anti-allergy (IL-4 antagonist)
Plasminogen activators e.g. Prourokinase	Erythrocytes, vascular endothelium	Prevention of venous thrombosis
Leptin	Choroid plexus, Hypothalamus	Weight loss (agonist)
Complement inhibitors*	Vascular endothelium, Myocytes, Erythrocytes, Lymphocytes	Ischaemic injury, transplantation, inflammation
scFv antibody against cytokines (IL-1, IL-, IL-5, IL-6)	Eosinophils	Asthma, allergic disease
Protein C	Vascular endothelium	Prevention of venous thrombosis
Antibodies against CD4, B7/CD28, CD3/TCR,	Lymphocytes	Immunosuppression

*CR1 (CD35)
DAF (CD55)
MCP (CD46)
CD59
Factor H
C4 binding protein
and hybrids or muteins thereof such as CR1-CD59 chimeras (S.G.El Feki and D.T.Fearon Molecular Immunology **33** (supp 1). p 57, 1996)

The derivative preferably comprises two to eight, more preferably two to four membrane binding elements.

Membrane binding elements are preferably selected from: fatty acid derivatives such as fatty acyl groups; basic amino acid sequences; ligands of known integral membrane proteins; sequences derived from the complementarity-determining region of monoclonal antibodies raised against epitopes of membrane proteins; membrane binding sequences identified through screening of random chemical libraries.

The selection of suitable combination of membrane binding elements will be guided by the nature of the target cell membrane or components thereof.

Suitable fatty acid derivatives include myristoyl (12 methylene units) which is insufficiently large or hydrophobic to permit high affinity binding to membranes. Studies with myristoylated peptides (eg R.M.Peitzsch & S.McLaughlin, Biochemistry, 32, 10436-10443, 1993)) have shown that they have effective dissociation constants with model lipid systems of ~10^{-4} M and around 10 of the 12 methylene groups are buried in the lipid bilayer. Thus, aliphatic acyl groups with between about 8 and 18 methylene units, preferably 10-14, are suitable membrane binding elements. Other examples of suitable fatty acid derivatives include long-chain (8-18, preferably 10-14 methylene) aliphatic amines and thiols, steroid and farnesyl derivatives.

Membrane binding has been found to be associated with limited (single-site) modification with fatty acyl groups when combined with a cluster of basic aminoacids in the protein sequence which may interact with acidic phospholipid head groups and provide the additional energy to target membrane binding. This combination of effects has been termed the 'myristoyl-electrostatic switch' (S.McLaughlin and A.Aderem, TIBS, 20,272-276, 1994; J.F.Hancock *et al*, Cell, 63, 133-139,1990). Thus, a further example of suitable membrane binding elements are basic aminoacid sequences such as those found in proteins such as *Ras* and MARCKS (myristoylated alanine-rich C-kinase substrate, P.J. Blackshear, J. Biol. Chem., 268, 1501-1504, 1993) which mediate the electrostatic 'switch' through reversible phosphorylation of serine residues within the sequence and a concomitant neutralisation of the net positive charge. Such sequences

include but are not restricted to consecutive sequences of Lysine and Arginine such as (Lys)n where n is between 3 and 10, preferably 4 to 7.

Suitable examples of amino acid sequences comprising basic amino acids include:

i) DGPKKKKKKSPSKSSG

ii) GSSKSPSKKKKKKPGD

iii) SPSNETPKKKKKRFSFKKSG

(N-terminus on left)

Sequences i) to iii) are examples of electrostatic switch sequences.

Examples of amino acid sequences include RGD-containing peptides such as GRGDSP which are ligands for the $\alpha_{IIb}\beta_3$ integrin of human platelet membranes. Further examples of such sequences include those known to be involved in interactions between membrane proteins such as receptors and the major histocompatibility complex. An example of such a membrane protein ligand is the sequence GNEQSFRVDLRTLLRYA which has been shown to bind to the major histocompatibility complex class 1 protein (MHC-1) with moderate affinity (L.Olsson *et al*, Proc. Natl .Acad.Sci.USA. 91, 9086-909, 1994).

An example of a ligand for an integral membrane protein is the carbohydrate ligand Sialyl Lewis^x which has been identified as a ligand for the integral membrane protein ELAM-1 (M.L.Phillips *et al*, Science, 250, 1130-1132, 1990 & G.Walz *et al*, *Ibid*, 250, 1132-1135,1990).

Sequences derived from the complementarity-determining regions of monoclonal antibodies raised against epitopes within membrane proteins (see, for example, J.W.Smith *et al*, J.Biol.Chem. 270, 30486-30490, 1995) are also suitable membrane binding elements, as are binding sequences from random chemical libraries such as those generated in a phage display format and selected by biopanning operations *in vitro* (G.F.Smith and J.K.Scott, Methods in Enzymology, 217H, 228-257,1993) or *in vivo* (R.Pasqualini & E.Ruoslahti, Nature, 380, 364-366, 1996).

Optionally, conditional dissociation from the membrane may be incorporated into derivatives of the invention using mechanisms such as pH sensitivity (electrostatic switches); regulation through metal ion binding (using endogenous Ca^{++}, Zn^{++} and incorporation of ion binding sites in membrane binding elements) and protease cleavage (e g plasminolysis of lysine-rich membrane binding sequences to release and activate prourokinase)

Preferred derivatives of this invention have the following structure:

(P)-Q-(W)$_n$-X

P is the soluble polypeptide,

each **L** is independently a flexible linker group,

each **W** is independently a peptidic membrane binding element,

n is an integer of 1 or more and

X is a peptidic or non-peptidic membrane-binding entity which may be covalently linked to any **W**.

Peptidic membrane binding elements are preferably located sequentially either at the N or C terminus of the soluble polypeptide and are preferably 8 to 20 amino acids long. The amino acid sequences are linked to one another and to the soluble peptide by linker groups which are preferably selected from hydrophilic and/or flexible aminoacid sequences of 4 to 20 aminoacids; linear hydrophilic synthetic polymers; and chemical bridging groups.

Peptide linkages may be made chemically or biosynthetically by expression of appropriate coding DNA sequences. Non peptide linkages may be made chemically or enzymatically by post-translational modification.

The polypeptide portion of the derivatives of the invention may be prepared by expression in suitable hosts of modified genes encoding the soluble polypeptide of interest plus one or more peptidic membrane binding elements and optional residues such as cysteine to introduce linking groups to facilitate post translational derivatisation with additional membrane binding elements.

In a further aspect, therefore, the invention provides a process for preparing a derivative according to the invention which process comprises expressing DNA encoding the polypeptide portion of said derivative in a recombinant host cell and recovering the product and thereafter post translationally modifying the polypeptide to chemically introduce membrane binding elements.

In particular, the recombinant aspect of the process may comprise the steps of:

i) preparing a replicable expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes said polypeptide portion;

ii) transforming a host cell with said vector;

iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said polypeptide; and

iv) recovering said polypeptide.

Where the polypeptide portion is novel, the DNA polymer comprising a nucleotide sequence that encodes the polypeptide portion as well as the polypeptide portion itself and S-derivatives thereof, also form part of the invention.

The recombinant process of the invention may be performed by conventional recombinant techniques such as described in Sambrook *et al*., Molecular Cloning : A laboratory manual 2nd Edition. Cold Spring Harbor Laboratory Press (1989) and DNA Cloning vols I, II and III (D. M. Glover ed., IRL Press Ltd).

The invention also provides a process for preparing the DNA polymer by the condensation of appropriate mono-, di- or oligomeric nucleotide units.

The preparation may be carried out chemically, enzymatically, or by a combination of the two methods, *in vitro* or *in vivo* as appropriate. Thus, the DNA polymer may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts *et al*., in Biochemistry 1985, 24, 5090-5098.

The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerisation, or by a combination of these methods.

Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase 1 (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less.

Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to 37°C, generally in a volume of 50µl or less.

The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J.Gait, H.W.D. Matthes M. Singh, B.S. Sproat and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams *et al* , Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McManus and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and

The DNA polymer is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the polypeptide.

The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences.

The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired product. The design of a suitable strategy for the construction of the DNA molecule coding for the polypeptide is a routine matter for the skilled worker in the art.

In particular, consideration may be given to the codon usage of the particular host cell. The codons may be optimised for high level expression in *E. coli* using the principles set out in Devereux *et al.*, (1984) Nucl. Acid Res., **12**, 387.

The expression of the DNA polymer encoding the polypeptide in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. Novel expression vectors also form part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment, encode the polypeptide, under ligating conditions.

The ligation of the linear segment and more than one DNA molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired. The choice of vector will be determined in part by the host cell, which may be prokaryotic, such as *E. coli*, or eukaryotic, such as mouse C127, mouse myeloma, chinese hamster ovary, fungi e.g. filamentous fungi or unicellular 'yeast' or an insect cell such as Drosophila. The host cell may also be in a transgenic animal. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses derived from, for example, baculoviruses or vaccinia.

The DNA polymer may be assembled into vectors designed for isolation of stable transformed mammalian cell lines expressing the fragment e.g. bovine papillomavirus vectors in mouse C127 cells, or amplified vectors in chinese hamster ovary cells (DNA Cloning Vol. II D.M. Glover ed. IRL Press 1985; Kaufman, R.J. *et al.*, Molecular and Cellular Biology 5, 1750-1759, 1985; Pavlakis G.N. and Hamer, D.H. Proceedings of the National Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V. *et al.*,European Patent Application No. 0093619, 1983).

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Sambrook *et al.*,cited above. Polymerisation and ligation may be performed as described above for the preparation of the DNA polymer. Digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20°-70°C, generally in a volume of 50µl or less with 0.1-10µg DNA.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Sambrook *et al.*, cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E.coli*, may be treated with a solution of $CaCl_2$ (Cohen *et al.*,Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol or by electroporation as for example described by Bio-Rad Laboratories, Richmond, California, USA, manufacturers of an electroporator. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells or by using cationic liposomes.

The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Sambrook *et al.*, and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The protein product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial such as *E. coli* and the protein is expressed intracellularly, it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product is usually isolated from the nutrient medium.

Where the host cell is bacterial, such as *E. coli*, the product obtained from the culture may require folding for optimum functional activity. This is most likely if the protein is expressed as inclusion bodies. There are a number of aspects of the isolation

removal of contaminating *E. coli* proteins by specifically isolating the inclusion bodies and the subsequent additional purification prior to folding are important aspects of the procedure.

The folding process is carried out in such a way as to minimise aggregation of intermediate-folded states of the polypeptide. Thus, careful consideration needs to be given to, among others, the salt type and concentration, temperature, protein concentration, redox buffer concentrations and duration of folding. The exact condition for any given polypeptide generally cannot be predicted and must be determined by experiment.

There are numerous methods available for the folding of proteins from inclusion bodies and these are known to the skilled worker in this field. The methods generally involve breaking all the disulphide bonds in the inclusion body, for example with 50mM 2-mercaptoethanol, in the presence of a high concentration of denaturant such as 8M urea or 6M guanidine hydrochloride. The next step is to remove these agents to allow folding of the proteins to occur. Formation of the disulphide bridges requires an oxidising environment and this may be provided in a number of ways, for example by air, or by incorporating a suitable redox system, for example a mixture of reduced and oxidised glutathione.

Preferably, the inclusion body is solubilised using 8M urea, in the presence of mercaptoethanol, and protein is folded, after initial removal of contaminating proteins, by addition of cold buffer. Suitable buffers may be identified using the techniques described in I.Dodd *et al.* 'Perspectives in Protein Engineering and Complementary Technologies', Mayflower Publications, 66-69, 1995. A suitable buffer for many of the SCR constructs described herein is 20mM ethanolamine containing 1mM reduced glutathione and 0.5mM oxidised glutathione. The folding is preferably carried out at a temperature in the range 1 to 5°C over a period of 1 to 4 days.

If any precipitation or aggregation is observed, the aggregated protein can be removed in a number of ways, for example by centrifugation or by treatment with precipitants such as ammonium sulphate. Where either of these procedures are adopted, monomeric polypeptide is the major soluble product.

If the bacterial cell secretes the protein, folding is not usually necessary.

The polypeptide portion of the derivative of the invention may include a C-terminal cysteine to facilitate post translational modification. Expression in a bacterial system is preferred for proteins of moderate size (up to ~70kDa) and with <~8 disulphide bridges. More complex proteins for which a free terminal Cys could cause refolding or stability problems may require stable expression in mammalian cell lines (especially CHO). This will also be needed if a carbohydrate membrane binding element is to be

introduced post-translationally. The use of insect cells infected with recombinant baculovirus encoding the polypeptide portion is also a useful general method for preparing more complex proteins and will be preferred when it is desired to carry out certain post-translational processes (such as palmitoylation) biosynthetically (see for example, M.J.Page *et al* J.Biol.Chem. 264, 19147-19154, 1989)

A preferred method of handling proteins C-terminally derivatised with cysteine is as a mixed disulphide with mercaptoethanol or glutathione or as the 2-nitro, 5-carboxyphenyl thio- derivative as generally described below in Methods.

Peptide membrane binding elements may be prepared using standard solid state synthesis such as the Merrifield method and this method can be adapted to incorporate required non-peptide membrane binding elements such as N-acyl groups derived from myristic or palmitic acids at the N terminus of the peptide. In addition activation of an amino acid residue for subsequent linkage to a protein can be achieved during chemical synthesis of such membrane binding elements. Examples of such activations include formation of the mixed 2-pyridyl disulphide with a cysteine thiol or incorporation of an N-haloacetyl group. Peptides can optionally be prepared as the C-terminal amide.

The invention also provides a peptide membrane binding element comprising one or more derivatisations selected from:

a terminal cysteine residue optionally activated at the thiol group;

an N-haloacetyl group (where halo signifies chlorine, bromine or iodine) located at the N-terminus of the the peptide or at an ε-amino group of a lysine residue;

an amide group at the C-terminus; and

a fatty acid N-acyl group at the N-terminus or at an ε-amino group of a lysine residue.

Chemical bridging groups include those described in EP0109653 and EP0152736. The bridging group is generally of the formula:

$$-A-R-B- \qquad (I)$$

in which each of A and B, which may be the same or different, represents -CO-, $-C(=NH_2^+)-$, maleimido, -S- or a bond and R is a bond or a linking group containing one or more $-(CH_2)-$ or meta- or para- disubstituted phenyl units.

Where the polypeptide portion of the derivative of the invention and a peptidic membrane binding element both include a C-terminal cysteine the chemical bridging group will take the form -S-S-. The bridge is generated by conventional disulphide exchange chemistry, by activating a thiol on one polypeptide and reacting the activated

which form intermediate mixed disulphides capable of further reaction with thiols to give stable disulphide linkages.

R may include moieties which interact with water to maintain the water solubility of the linkage and suitable moieties include -CO-NH-, -CO-NMe-, -S-S-, -CH(OH)-, $-SO_2-$, $-CO_2-$, $-(CH_2CH_2-O)_m-$ and -CH(COOH)- where m is an integer of 2 or more.

Examples of R include $-(CH_2)_r-$, $-(CH_2)_p-S-S-(CH_2)_q-$ and $-(CH_2)_p-CH(OH)-CH(OH)-(CH_2)_q-$, in which r is an integer of at least 2, preferably at least 4 and p and q are independently integers of at least 2.

The bridging group of formula (I) may be derived from a linking agent of formula (II):

$$X-R_1-Y \qquad (II)$$

in which R_1 is a linking group containing one or more $-(CH_2)-$ units and X and Y are functional groups reactable with surface amino acid groups, preferably a lysine or cysteine group, or the N-terminal amino group, or a protein attachment group.

Preferred agents are those where X and Y are different, known as heterobifunctional agents. Each end of the agent molecule is reacted in turn with each polypeptide to be linked in separate reactions. Examples of heterobifunctional agents of formula (II) include:

N-succinimidyl 3-(2-pyridyldithio) propionate

succinimidyl 4-(N-maleimido) caproate

3-(2-pyridyl) methyl propionimidate hydrochloride

In each case Y is capable of reacting with a thiol group on a polypeptide, which may be a native thiol or one introduced as a protein attachment group.

The protein attachment group is a functionality derived by modification of a polypeptide with a reagent specific for one or more amino acid side chains, and which contains a group capable of reacting with a cleavable section on the other polypeptide. An example of a protein attachment group is a thiol group. An example of a cleavable section is a disulphide bond. Alternatively the cleavable section may comprise an α, β dihydroxy function.

As an example, the introduction of a free thiol function by reaction of a polypeptide with 2-iminothiolane, N-succinimidyl 3-(2-pyridyldithio) propionate (with subsequent reduction) or N-acetyl homocysteine thiolactone will permit coupling of the protein attachment group with a thiol-reactive B structure. Alternatively, the protein attachment group can contain a thiol-reactive entity such as the 6-maleimidohexyl group or a 2-pyridyl-dithio group which can react with a free thiol in X. Preferably, the protein attachment group is derived from protein modifying agents such as 2-iminothiolane that react with lysine ε-amino groups in proteins.

When X represents a group capable of reacting directly with the amino acid side chain of a protein, it is preferably an N-succinimidyl group. When X represents a group capable of reacting with a protein attachment group, it is preferably a pyridylthio group.

In the above processes, modification of a polypeptide to introduce a protein attachment group is preferably carried out in aqueous buffered media at a pH between 3.0 and 9.0 depending on the reagent used. For a preferred reagent, 2-iminothiolane, the pH is preferably 6.5-8.5. The concentration of polypeptide is preferably high (> 10mg/ml) and the modifying reagent is used in a moderate (1.1- to 5-fold) molar excess, depending on the reactivity of the reagent. The temperature and duration of reaction are preferably in the range 0^{o}-40^{o}C and 10 minutes to 7 days. The extent of modification of the polypeptide may be determined by assaying for attachment groups introduced.

Such assays may be standard protein chemical techniques such as titration with 5,5'-dithiobis-(2-nitrobenzoic acid). Preferably, 0.5-3.0 moles of protein attachment group will be introduced on average per mole of polypeptide. The modified polypeptide may be separated from excess modifying agents by standard techniques such as dialysis, ultrafiltration, gel filtration and solvent or salt precipitation. The intermediate material may be stored in frozen solution or lyophilised.

Where a protein attachment group is introduced in this way, the bridging group (I) will be formed from a reaction of the linking agent (II) and the protein attachment group.

The polypeptides to be linked are reacted separately with the linking agent or the reagent for introducing a protein attachment group by typically adding an excess of the reagent to the polypeptide, usually in a neutral or moderately alkaline buffer, and after reaction removing low molecular weight materials by gel filtration or dialysis. The precise conditions of pH, temperature, buffer and reaction time will depend on the nature of the reagent used and the polypeptide to be modified. The polypeptide linkage reaction is preferably carried out by mixing the modified polypeptides in neutral buffer in an equimolar ratio. Other reaction conditions e.g. time and temperature, should be chosen to obtain the desired degree of linkage. If thiol exchange reactions are involved, the reaction should preferably be carried out under an atmosphere of nitrogen. Preferably, UV-active products are produced (eg from the release of pyridine 2-thione from 2-pyridyl dithio derivatives) so that coupling can be monitored.

After the linkage reaction, the polypeptide conjugate can be isolated by a number of chromatographic procedures such as gel filtration, ion-exchange chromatography,

The conjugate may be characterised by a number of techniques including low pressure or high performance gel filtration, SDS polyacrylamide gel electrophoresis or isoelectric focussing.

Membrane binding elements which are fatty acid derivatives are attached post translationally to a peptidic membrane binding element, preferably at the terminus of the polypetide chain. Preferably, where the recombinant polypeptide portion of the derivative of the invention contains the peptidic membrane binding element, it has a unique cysteine for coupling to the fatty acid derivative. Where the recombinant polypeptide has a cysteine residue, a thiol-derivative of the fatty acid is added to the refolded recombinant protein at a late stage in purification (but not necessarily the final stage) and at a reagent concentration preferably below the critical micelle concentration. One of the fatty acid derivative and the recombinant peptide will have the thiol group activated as described above for thiol interchange reactions. The fatty acid derivative is preferably a fatty acyl derivative of an aminoC_{2-6}alkane thiol (optionally C-substituted) such as N-(2-myristoyl) aminoethanethiol or N-myristoyl L-cysteine.

Suitable examples of hydrophilic synthetic polymers include polyethyleneglycol (PEG), preferably α,ω functionalised derivatives, more preferably α-amino , ω-carboxy-PEG of molecular weight between 400 and 5000 daltons which are linked to the polypeptide for example by solid-phase synthesis methods (amino group derivatisation) or by thiol-interchange chemistry.

Membrane binding elements derived from ligands of known integral membrane proteins, either amino acid sequences or carbohydrates, may be generated by post-translational modification using the glycosylation pathways of eukaryotic cells targeted to N-linked glycosylation sites in the peptide sequence.

Convenient generic final stage purification strategies are hydrophobic interaction chromatography (HIC) on C2-C8 media and cation exchange chromatography for separation of derivatised and underivatised proteins into which a hydrophobic-electrostatic switch combination has been inserted.

The derivatives of this invention are preferably administered as pharmaceutical compositions.

Accordingly, the present invention also provides a pharmaceutical composition comprising a derivative of the invention in combination with a pharmaceutically acceptable carrier.

The compositions according to the invention may be formulated in accordance with routine procedures for administration by any route, such as oral, topical, parenteral, sublingual or transdermal or by inhalation. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, creams or liquid preparations, such as oral

or sterile parenteral solutions or suspensions or in the form of a spray, aerosol or other conventional method for inhalation.

The topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, eye ointments and eye or ear drops, impregnated dressings and aerosols, and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams.

The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

Tablets and capsules for oral administration may be in unit dose presentation form, and may contain conventional excipients such as binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, or polyvinylpyrollidone; fillers, for example lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tabletting lubricants, for example magnesium stearate, talc, polyethylene glycol or silica; disintegrants, for example potato starch; or acceptable wetting agents such as sodium lauryl sulphate. Tablets may also contain agents for the stablisation of polypeptide drugs against proteolysis and absorbtion-enhancing agents for macromolecules. The tablets may be coated according to methods well known in normal pharmaceutical practice.

Suppositories will contain conventional suppository bases, e.g. cocoa-butter or other glyceride.

For parenteral administration, fluid unit dosage forms are prepared utilizing the compound and a sterile vehicle, water being preferred. The compound, depending on the vehicle and concentration used, is dissolved in the vehicle. In preparing solutions the compound can be dissolved in water for injection and filter sterilised before filling into a suitable vial or ampoule and sealing.

Parenteral formulations may include sustained-release systems such as encapsulation within microspheres of biodegradable polymers such as poly-lactic co-glycolic acid.

Advantageously, agents such as a local anaesthetic, preservative and buffering agents can be dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. The dry lyophilized powder is then sealed in the vial and an accompanying vial of water for injection may be supplied to reconstitute the liquid prior to use. Advantageously, a

Compositions of this invention may also suitably be presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns for example diameters in the range of 1-50 microns, 1-10 microns or 1-5 microns. Where appropriate, small amounts of anti-asthmatics and bronchodilators, for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included.

Microfine powder formulations may suitably be administered in an aerosol as a metered dose or by means of a suitable breath-activated device.

Suitable metered dose aerosol formulations comprise conventional propellants, cosolvents, such as ethanol, surfactants such as oleyl alcohol, lubricants such as oleyl alcohol, desiccants such as calcium sulphate and density modifiers such as sodium chloride.

Suitable solutions for a nebulizer are isotonic sterilised solutions, optionally buffered, at for example between pH 4-7, containing up to 20mg ml^{-1} of compound but more generally 0.1 to 10mg ml^{-1}, for use with standard nebulisation equipment.

The quantity of material administered will depend upon the potency of the derivative and the nature of the complaint be decided according to the circumstances by the physician supervising treatment. However, in general, an effective amount of the polypeptide for the treatment of a disease or disorder is in the dose range of 0.01-100mg/kg per day, preferably 0.1mg-10mg/kg per day, administered in up to five doses or by infusion.

No adverse toxicological effects are indicated with the compounds of the invention within the above described dosage range.

The invention also provides a derivative of the invention for use as a medicament.

In one preferred aspect the present invention relates to derivatives for use in the therapy of disorders involving complement activity and various inflammatory and immune disorders.

In this preferred aspect the soluble polypeptide which is derivatised in accordance with the invention is a soluble CR1 polypeptide fragment.

Constituting about 10% of the globulins in normal serum, the complement system is composed of many different proteins that are important in the immune system's response to foreign antigens. The complement system becomes activated when its primary components are cleaved and the products alone or with other proteins, activate

additional complement proteins resulting in a proteolytic cascade. Activation of the complement system leads to a variety of responses including increased vascular permeability, chemotaxis of phagocytic cells, activation of inflammatory cells, opsonization of foreign particles, direct killing of cells and tissue damage. Activation of the complement system may be triggered by antigen-antibody complexes (the classical pathway) or, for example, by lipopolysaccharides present in cell walls of pathogenic bacteria (the alternative pathway).

Complement receptor type 1 (CR1) has been shown to be present on the membranes of erythrocytes, monocytes/macrophages, granulocytes, B cells, some T cells, splenic follicular dendritic cells, and glomerular podocytes. CR1 binds to the complement components C3b and C4b and has also been referred to as the C3b/C4b receptor. The structural organisation and primary sequence of one allotype of CR1 is known (Klickstein *et al.*, 1987, J. Exp. Med. 165:1095-1112, Klickstein *et al.*, 1988, J. Exp. Med. 168:1699-1717; Hourcade *et al.*,1988, J. Exp. Med. 168:1255-1270, WO 89/09220, WO 91/05047). It is composed of 30 short consensus repeats (SCRs) that each contain around 60-70 amino acids. In each SCR, around 29 of the average 65 amino acids are conserved. Each SCR has been proposed to form a three dimensional triple loop structure through disulphide linkages with the third and first and the fourth and second half-cystines in disulphide bonds. CR1 is further arranged as 4 long homologous repeats (LHRs) of 7 SCRs each. Following a leader sequence, the CR1 molecule consists of the N-terminal LHR-A, the next two repeats, LHR-B and LHR-C, and the most C-terminal LHR-D followed by 2 additional SCRs, a 25 residue putative transmembrane region and a 43 residue cytoplasmic tail.

Based on the mature CR1 molecule having a predicted N-terminal glutamine residue, hereinafter designated as residue 1, the first four SCR domains of LHR-A are defined herein as consisting of residues 2-58, 63-120, 125-191 and 197-252, respectively, of mature CR1.

Several soluble fragments of CR1 have been generated via recombinant DNA procedures by eliminating the transmembrane region from the DNAs being expressed (WO 89/09220, WO 91/05047). The soluble CR1 fragments were functionally active, bound C3b and/or C4b and demonstrated Factor I cofactor activity depending upon the regions they contained. Such constructs inhibited *in vitro* complement-related functions such as neutrophil oxidative burst, complement mediated hemolysis, and C3a and C5a production. A particular soluble construct, sCR1/pBSCR1c, also demonstrated *in vivo*

Dupe, R. *et al.* Thrombosis & Haemostasis (1991) 65(6) 695.) and extended survival rates following transplantation (Pruitt & Bollinger, 1991, J. Surg. Res 50:350; Pruitt *et al.*, 1991 Transplantation 52; 868). Furthermore, co-formulation of sCR1/pBSCR1c with p-anisoylated human plasminogen-streptokinase-activator complex (APSAC) resulted in similar anti-haemolytic activity as sCR1 alone, indicating that the combination of the complement inhibitor sCR1 with a thrombolytic agent was feasible (WO 91/05047).

The soluble CR1 polypeptide fragment encoded by sCR1/pBSCR1c, residues 1-1929 of CR1, may be derivatised in accordance with the invention.

Soluble polypeptides corresponding to part of CR1 have been found to possess functional complement inhibitory, including anti-haemolytic, activity. WO94/00571 discloses soluble polypeptides comprising, in sequence, one to four short consensus repeats (SCR) selected from SCR 1, 2, 3 and 4 of long homologous repeat A (LHR-A) as the only structurally and functionally intact SCR domains of CR1 and including at least SCR3.

In preferred aspects, the polypeptide comprises, in sequence, SCR 1, 2, 3 and 4 of LHR-A or SCR 1, 2 and 3 of LHR-A as the only structurally and functionally intact SCR domains of CR1.

In one aspect, the polypeptides may be represented symbolically as follows:

$$NH_2\text{-}V^1\text{-SCR1-}W^1\text{-SCR2-}X^1\text{-SCR3-}Y^1\text{-OH} \qquad (A)$$

in which SCR1 represents residues 2-58 of mature CR1, SCR2 represents residues 63-120 of mature CR1, SCR3 represents residues 125-191 of mature CR1, and V^1, W^1, X^1 and Y^1 represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (I), W^1, X^1 and Y^1 represent residues 59-62, 121-124 and 192-196, respectively, of mature CR1 and V^1 represents residue 1 of mature CR1 optionally linked via its N-terminus to methionine.

In another aspect the polypeptides may be represented symbolically as follows:

$$NH_2\text{-}V^2\text{-SCR1-}W^2\text{-SCR2-}X^2\text{-SCR3-}Y^2\text{-SCR4-}Z^2\text{OH} \qquad (B)$$

in which SCR1, SCR2 and SCR3 are as hereinbefore defined, SCR4 represents residues 197-252 of mature CR1 and V^2, W^2, X^2, Y^2 and Z^2 represents bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In preferred embodiments of formula (II), W^2, X^2, Y^2 and Z^2 represent residues 59-62, 121-124, 192-196, and residues 253 respectively, of mature CR1 and V^2 represents residue 1 of mature CR1 optionally linked via its N-terminus to methionine.

In one particular embodiment of formula (B) arginine 235 is replaced by histidine.

In the preferred embodiment of formula (B), residue 235 is arginine.

In one further aspect, the polypeptide may be represented symbolically as follows:

$$NH_2\text{-}X^3\text{-}SCR3\text{-}Y^3\text{-}OH \qquad (C)$$

in which SCR3 is as hereinbefore defined and X^3 and Y^3 represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (C) X^3 represents amino acids 122-124 of mature CR1 optionally linked to methionine at its N-terminus and Y^4 represents amino acids 192-196 of mature CR1.

In another further aspect, the polypeptide may be represented symbolically as follows:

$$NH_2\text{-}X^4\text{-}SCR3\text{-}Y^4\text{-}SCR4\text{-}Z^4\text{-}OH \qquad (D)$$

in which SCR3 and SCR4 are as hereinbefore defined and X^4, Y^4 and Z^4 represent bonds or short linking sequences of amino acids, preferably 1 to 5 residues in length and which are preferably derived from native interdomain sequences in CR1.

In a preferred embodiment of formula (D) X^4 represents amino acids 122-124 of mature CR1 optionally linked to methionine at its N-terminus and Y^4 and Z^4 represent amino acids 192-196 and 253 respectively of mature CR1.

The soluble CR1 polypeptide is derivatised in accordance with the invention by any convenient strategy from those outlined above. In a preferred embodiment the soluble CR1 polypeptide consists of residues 1-196 of CR1 and with an N-terminal methionine and the derivative comprises a myristoyl group and a polypeptide sequence selected from

DGPKKKKKKSPSKSSG

and

GSSKSPSKKKKKKPGD

(N-terminus on left).

The soluble CR1 polypeptide derivative of this invention is useful in the treatment of many complement-mediated or complement-related diseases and disorders including, but not limited to, those listed below.

Diseases and Disorders Involving Complement

stroke
Guillain Barré Syndrome
traumatic brain injury
Parkinson's disease
allergic encephalitis
Alzheimer's disease

Disorders of Inappropriate or Undesirable Complement Activation
haemodialysis complications
hyperacute allograft rejection
xenograft rejection
corneal graft rejection
interleukin-2 induced toxicity during IL-2 therapy
paroxysmal nocturnal haemoglobinuria

Inflammatory Disorders
inflammation of autoimmune diseases
Crohn's Disease
adult respiratory distress syndrome
thermal injury including burns or frostbite
uveitis
psoriasis
asthma
acute pancreatitis

Post-Ischemic Reperfusion Conditions
myocardial infarction
balloon angioplasty
atherosclerosis (cholesterol-induced) & restenosis
hypertension
post-pump syndrome in cardiopulmonary bypass or renal haemodialysis
renal ischemia
intestinal ischaemia

Infectious Diseases or Sepsis
multiple organ failure
septic shock

Immune Complex Disorders and Autoimmune Diseases

rheumatoid arthritis
systemic lupus erythematosus (SLE)
SLE nephritis
proliferative nephritis
glomerulonephritis
haemolytic anemia
myasthenia gravis

Reproductive Disorders

antibody- or complement-mediated infertility

Wound Healing

The present invention is also directed to a pharmaceutical composition for treating a disease or disorder associated with inflammation or inappropriate complement activation comprising a therapeutically effective amount of a soluble CR1 polypeptide derivative of this invention, and a pharmaceutically acceptable carrier or excipient.

The present invention also provides a method of treating a disease or disorder associated with inflammation or inappropriate complement activation comprising administering to a subject in need of such treatment a therapeutically effective amount of a a soluble CR1 polypeptide derivative of this invention.

In the above methods, the subject is preferably a human.

Further provided is the use of a soluble CR1 polypeptide derivative of this invention in the manufacture of a medicament for the treatment of a disease or disorder associated with inflammation or inappropriate complement activation.

In order to inhibit complement activation and, at the same time, provide thrombolytic therapy, the present invention provides compositions which further comprise a therapeutically active amount of a thrombolytic agent. An effective amount of a thrombolytic agent is in the dose range of 0.01-10mg/kg; preferably 0.1-5mg/kg. Preferred thrombolytic agents include, but are not limited to, streptokinase, human tissue type plasminogen activator and urokinase molecules and derivatives, fragments or conjugates thereof. The thrombolytic agents may comprise one or more chains that may

thrombolytic agents may also comprise muteins of plasminogen activators (EP-A-0207589). In a preferred embodiment, the thrombolytic agent may comprise a reversibly blocked *in vitro* fibrinolytic enzyme as described in U.S. Patent No. 4,285,932. A most preferred enzyme is the p-anisoyl plasminogen-streptokinase activator complex, anistreplase as described in U.S. Patent No. 4,808,405 (Monk *et al.*, 1987, Drugs 34:25-49).

Routes of administration for the individual or combined therapeutic compositions of the present invention include standard routes, such as, for example, intravenous infusion or bolus injection. Active complement inhibitors and thrombolytic agents may be administered together or sequentially, in any order.

The present invention also provides a method for treating a thrombotic condition, in particular acute myocardial infarction, in a human or non-human animal. This method comprises administering to a human or animal in need of this treatment an effective amount of a soluble CR1 polypeptide derivative according to this invention and an effective amount of a thrombolytic agent.

Also provided is the use of a soluble CR1 polypeptide derivative of this invention and a thrombolytic agent in the manufacture of a medicament for the treatment of a thrombotic condition in a human or animal. Such methods and uses may be carried out as described in WO 91/05047.

This invention further provides a method for treating adult respiratory distress syndrome (ARDS) in a human or non-human animal. This method comprises administering to the patient an effective amount of a soluble CR1 polypeptide derivative according to this invention.

The invention also provides a method of delaying hyperacute allograft or hyperacute xenograft rejection in a human or non-human animal which receives a transplant by administering an effective amount of a soluble CR1 polypeptide derivative according to this invention. Such administration may be to the patient or by application to the transplant prior to implantation.

The invention yet further provides a method of treating wounds in a human or non-human animal by administering by either topical or parenteral e.g. intravenous routes, an effective amount of a soluble CR1 polypeptide derivative according to this invention.

The following Methods and Examples illustrate the invention.

GENERAL METHODS USED IN EXAMPLES

(i) DNA Cleavage

Cleavage of DNA by restriction endonucleases was carried out according to the manufacturer's instructions using supplied buffers. Double digests were carried out simultaneously if the buffer conditions were suitable for both enzymes. Otherwise double digests were carried out sequentially where the enzyme requiring the lowest salt condition was added first to the digest. Once the digest was complete the salt concentration was altered and the second enzyme added.

(ii) DNA ligation

Ligations were carried out using T4 DNA ligase purchased from Promega, as described in Sambrook *et al*, (1989) Molecular Cloning: A Laboratory Manual 2nd Edition. Cold Spring Harbour Laboratory Press.

(iii) Plasmid isolation

Plasmid isolation was using Promega Wizard™ *Plus* Minipreps or Qiagen Plasmid Maxi kit according to the manufacturer's instructions.

(iv) DNA fragment isolation

DNA fragments were excised from agarose gels and DNA extracted using the QIAEX gel extraction kit or Qiaquick gel extraction kit according to the manufacturer's instructions (QIAGEN Inc., USA).

(v) Introduction of DNA into *E. coli*

Plasmids were transformed into *E. coli* BL21(DE3) (Studier and Moffat, (1986), *J. Mol. Biol* 189:113) that had been made competent using calcium chloride as described in Sambrook *et al*, (1989). *E. coli* JM109 was purchased as a frozen competent culture from Promega.

(vi) DNA sequencing

Plasmid DNA was sequenced on a Vistra DNA Labstation 625. The sequencing chemistry was performed using Amersham International's Thermo Sequenase fluorescent dye-terminator cycle sequencing kit' (RPN 2435), in conjunction with their 'FMP

The sequences produced by the above procedure were analysed by a Perkin Elmer ABI Prism 377 DNA Sequencer. This is an electrophoretic technique using 36 cm x 0.2mm 4% acrylamide gels, the fluorescently labeled DNA fragments being detected by a charge coupled device camera according to the manufacturer's instructions.

(vii) Production of oligonucleotides

Oligonucleotides were purchased from Cruachem.

(viii) pBROC413

The plasmid pT7-7 [Tabor, S (1990), Current Protocols in Molecular Biology, F. A. Ausubel, Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl,eds.] pp.16.2.1-16.2.11, Greene Publishing and Wiley-Interscience,New York.] contains DNA corresponding to nucleotides 2065-4362 of pBR322 and like pBR322 can be mobilized by a conjugative plasmid in the presence of a third plasmid ColK. A mobility protein encoded by ColK acts on the *nic* site at nucleotide 2254 of pBR322 initiating mobilization from this point. pT7-7 was digested with *Lsp*I and *Bgl*II and the protruding 5' ends filled in with the Klenow fragment of DNA PolymeraseI. The plasmid DNA fragment was purified by agarose gel electrophoresis, the blunt ends ligated together and transformed into *E. coli* DH1 by electroporation using a Bio-Rad Gene Pulser and following the manufacturers recommended conditions. The resultant plasmid pBROC413 was identified by restriction enzyme analysis of plasmid DNA.

The deletion in pBROC413 from the *Lsp*I site immediately upstream of the f 10 promoter to the *Bgl*II site at nucleotide 434 of pT7-7 deletes the DNA corresponding to nucleotides 2065-2297 of pBR322. The *nic* site and adjacent sequences are therefore deleted making pBROC413 non mobilizable.

(ix) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE was carried out generally using the Novex system (British Biotechnology) according to the manufacturer's instructions. Prepacked gels of acrylamide concentrations 4 - 20% were most frequently used. Samples for electrophoresis, including protein molecular weight standards (LMW Kit, Pharmacia) were usually diluted in 1%(w/v)SDS - containing buffer (with or without 5%(v/v) 2-mercaptoethanol), and left at room temperature for about 0.5 h before application to the gel.

(x) Reduction of disulphides and modification of free thiols in proteins

There are a number of methods used for achieving the title goals. The specific considerations used here relate to the observation by mass spectrophotometry analysis that the product after refolding, concentration and further purification was the glutathione or 2-mercaptoethanol derivatised protein. Since the biological activity of the product was the same as the parent protein, it was believed that the modification was at the C-terminal free thiol. These derivatives are generally quite stable. In order to make them more reactive they need to be selectively reduced, with for example dithiothreitol (DTT) or with Tris (2-carboxyethyl) phosphine.HCl (TCEP) then optionally modified with a function which is moderately unstable. An example of the latter is Ellmans reagent (DTNB) which gives a mixed disulphide. In the case where treatment with DTNB is omitted, careful attention to experimental design is necessary to ensure that dimerisation of the free thiol-containing protein is minimised. Reference to the term 'selectively reduced' above means that reaction conditions eg. duration, temperature, molar ratios of reactants have to be carefully controlled so that disulphide bridges within the natural architecture of the protein are not reduced. All the reagents are commercially available eg. from Sigma or Pierce.

The following general examples illustrate the type of conditions that may be used and that are useful for the generation of free thiols and their optional modification. The specific reaction conditions to achieve optimal thiol reduction and/or modification are ideally determined for each protein batch.

TCEP may be prepared as a 20mM solution in 50mM Hepes (approx. pH 4.5) and may be stored at -40 degrees C. DTT may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40 degrees C. DTNB may be prepared at 10mM in sodium phosphate pH 7.0 and may be stored at -40 degrees C. All of the above reagents are typically used at molar equivalence or molar excess, the precise concentrations ideally identified experimentally. The duration and the temperature of the reaction are similarly determined experimentally. Generally the duration would be in the range 1 to 24 hours and the temperature would be in the range 2 to 30 degrees C. Excess reagent may be conveniently removed by buffer exchange, for example using Sephadex G25, into 0.1M sodium phosphate pH 7.0 or if the reagent can be tolerated during the following step the solution may be left untreated

EXAMPLES

Example 1 Preparation of N-(Myristoyl) 2-aminoethane thiol (MAET)

Myristoyl chloride (1.0 mmol)was added with vigorous stirring to ice-cooled dry pyridine (1.0ml), and followed immediately by N-hydroxysuccinimide (1.5 mmol).The mixture was stirred for 4h at ambient termperature (~23°C). 2-aminoethanethiol free base (1.1 mmol) was added as solid to the mixture and allowed to react for 6h at ambient temperature, followed by 3 days at 4°C. The product was treated with water (5ml), stirred for 1h at ambient and filtered, washing with cold water. The white solid was dissolved in dimethylsulphoxide and reprecipitated with water and then vacuum dried over phosphorous pentoxide. The final yield was 0.21g (~70%). Thiol titration using Ellman's reagent indicated that the product contained ~45% free thiol.

Example 2 Synthesis of Myristoyl/Electrostatic Switch Peptide Reagent 1 (MSWP-1)

N-(Myristoyl) -Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-(S-2-Thiopyridyl)Cys-NH_2

The peptide:

Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

(SEQ ID NO 5)

was prepared using solid phase synthesis via the general Fmoc/tBu strategy developed by Sheppard and Atherton (E.Atherton and R.C.Sheppard, Solid Phase Synthesis, IRL Press, Oxford, 1989). Kieselguhr-supported polydimethylacrylamide resin (Macrosorb 100) was used as the solid support and was derivatised with ethylene diamine.

Coupling reactions were carried out using N-α-Fmoc protected reagents pre-activated with N,N'-diisopropylcarbodiimide/ N-hydroxybenzotriazole (in 4-fold molar excess) with bromophenol blue monitoring. Fmoc cleavages used 20% piperidine in DMF. Reactions to assemble the peptide chain were carried out by repeated cycles of coupling and deprotection including the attachment of the modified Rink linkage reagent (p-[(R,S)-α-[1-(9H-fluoreny-9-yl-methoxyformamido | 2,4 dimethoxybenzyl]-phenoxyacetic acid) designed to yield a C-terminal amide on final cleavage. The side chain functionalities of the individual amino-acids were protected as follows:

Ser (tButyl), Lys (Boc), Asp (O-tButyl), Cys (Trityl).

On completion of the peptide assembly and with the peptide still attached to the resin, the myristoyl group was attached to the amino group of the N terminal glycine by direct coupling of myristic acid by the same activation procedure. This modified peptide

was then cleaved from the resin and the side-chain protecting groups removed at the same time by treatment with trifluoracetic acid containing 2.5% water and 2.5% triisopropyl silane.

The crude product was treated with 2,2' dithiopyridine in 0.01M ammonium acetate solution at pH 8-9 for approx. 2h, then acidified with acetic acid and purified by preparative high performance liquid chromatography (HPLC) in 0.1% trifluoracetic acid (TFA) /water and 0.1% TFA/acetonitrile as gradient component. After lyophilisation, the peptide was a white amorphous powder, soluble to at least 10mg/ml in dimethylsulphoxide. Fast atom bombardment mass spectrometry gave main peaks at m/e 2107.8, 2129.7 and 2145.8, corresponding to the monoprotonated, monosodiated and monopotassiated molecular ions of the peptide. The 2-thiopyridyl content of the peptide was measured by dissolving it to around 0.03mM to 0.2 mM in 0.1M Sodium Borate pH 8.0 and reducing by addition of dithiothreitol to 5mM. The change in optical density at 343nm was used to calculate the amount of pyridine 2-thione released using an extinction coefficient at this wavelength of 8080 cm^{-1} M^{-1} . This indicated that the peptide content was approximately 60% of the dry weight.

Example 3 Expression and isolation of [SCR1-3]-Cys (SEQ ID 6)

(a) Construction of plasmid pDB1030 encoding [SCR 1-3]-Cys

The plasmid coding for SCR1-3 of LHR-A of CR1, pDB1013-5 (patent application WO 94/00571) was digested with restriction endonucleases *Eco*RI and *Hin*dIII and the 2.2 kB plasmid band was isolated from an agarose gel using a Qiagen Qiaex DNA extraction kit according to the manufacturer's instructions. This is fragment 1. A second batch of pDB1013-5 was digested with *Ban*I and *Eco*RI and the 196 bp band was extracted from agarose as above. This is fragment 2. Two oligonucleotides, SEQ ID No.1 and , SEQ ID No.2, were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has a *Ban*I/*Eco*RI overhang and duplicates the sequence at the 3' end of pDB1013-5 but in addition contains a codon coding for cysteine just before the stop codon. This is fragment 3.

Fragments 1,2 and 3 were ligated with T4 DNA ligase in a single reaction to give pDB1030 . The ligated plasmid was transformed into competent *E. coli* JM109 purchased from Promega. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of

(b) **Expression of [SCR1-3]-Cys from pDB1030**

pDB1030 was transformed into calcium chloride competent *E. coli* BL21(DE3) and resultant colonies were isolated and checked for plasmid content. To express protein from pDB1030 in *E. coli* BL21(DE3), a single colony was inoculated into 10 ml LB-phosphate media (20g/L tryptone, 15g/L yeast extract, 0.8g/L NaCl, 0.2g/L Na_2HPO_4, 0.1g/L KH_2PO_4) containing 50ug/ml ampicillin. The culture was grown for 6 hours at 37°C, 230 r.p.m. before being used to inoculate 100 ml of the same media containing 50 ug/ml ampicillin. Growth was under the same conditions overnight. 25 ml of each culture were then used to inoculate 600 ml of the same media with 50 ug/ml ampicillin in 3 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at A_{600} nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours before harvesting by centrifugation at 8000 g/10 min. Pellet from 2L of culture was stored at -80°C

(c) **Isolation, refolding, purification and formulation of [SCR1-3]-Cys**

The methods described are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736.

i) **Isolation of solubilised inclusion bodies**

The frozen cell pellet of *E. coli* BL21(DE3) (pDB1030) was resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM PMSF pH 8.0 at a ratio of 33 ml for each litre of culture pellet. The suspension was transferred to a glass beaker surrounded by ice and sonicated (Heat systems - Ultrasonics W380; 50 x 50% pulse, pulse time = 5 sec.) for typically 3 - 6 minutes. The disrupted pellet was then frozen and stored at -80°C. Approx. 2 weeks later the sonicate was thawed and centrifuged at approx. 8000g for 20 min. The pellet was resuspended in 20 mM Tris/8M urea/1 mM EDTA/50 mM 2-mercaptoethanol pH 8.5 (200ml) at room temperature by vigorous swirling, then left for 1h at room temperature followed by overnight at 4° C.

ii) **Initial purification using SP-Sepharose**

To the viscous solution was added SP-Sepharose FF (approx. 30g wet weight) that had been water washed and suction-dried. The mixture was swirled vigorously and left static for 1-2h at room temperature. The supernatant was decanted, sampled and discarded. The remaining slurry was resuspended to a uniform suspension and poured into a glass jacket and allowed to settle into a packed bed. The column was equilibrated with 0.02M Tris/8M urea/0 05M 2-mercaptoethanol/0.001 M EDTA pH 8.5.at 4°C. When the A_{280} of the eluate had stabilised at baseline, the buffer was changed to equilibration buffer additionally containing 1M NaCl. A single A_{280} peak was eluted by the 1M NaCl-containing buffer; the volume was approx. 50ml. The protein

concentration of the solution was estimated by A_{280} determination using a molar extinction coefficient of 25000 cm^{-1} of a sample that had been buffer-exchanged (Sephadex G25) into 50 mM formic acid. This showed the product had a protein concentration of 1.6mg/ml. The solution was stored at -40°C.

iii) **Folding and further processing**

25ml of the SP-Sepharose-purified product was added gradually over a 1 min period to 780 ml freshly prepared, cold 0.02M ethanolamine/1mM EDTA with continuous swirling, and left static for 1 h/4°C. Reduced glutathione (GSH) was added to 1 mM and oxidised glutathione (GSSG) was added to 0.5 mM. The solution was clear and was left static approx 2-3°C for 3 d. The solution was then ultrafiltered using a YM10 membrane to a final retentate volume of about 35 ml; the retentate was slightly cloudy and had the appearance of a translucent solution. It was stored for 12 days at 4° C. It was then spun at 30 000g for 15 mins and the supernatant mixed with 9 vol. 0.1M NaH_2PO_4/1M $(NH_4)_2S0_4$ pH 7.0 (Buffer A) at room temperature and immediately centrifuged at 3000 rpm for 15 min. The supernatant was ultrafiltered (YM10) to about 4ml and then buffer-exchanged into 0.1M sodium phosphate pH 7.0 (5.0ml); this solution had a protein concentration of 1.7mg/ml by A280 analysis. It was treated with dithio bis nitrobenzoic acid (DTNB) (8-fold molar excess) for 30 min at room temperature. Free thiol content based on A412 measurement and an extinction coefficient (for the free thionitrobenzoate ion) of 13 600 was 6uM equivalent to only about 10% derivatisation to give Product A. The majority of the product was believed to be [SCR1-3]-Cys where the free C-terminal thiol was blocked by reaction with glutathione or 2-mercaptoethanol during the refolding stage.

(d) Alternative method for isolation, refolding, purification and formulation of [SCR1-3]-Cys

The method was identical to that described above, except that it more closely followed the procedures described in Dodd et al (op cit.). Notably, the ultrafiltered retentate post refolding was immediately treated with ammonium sulphate followed by clarification by centrifugation and Butyl Sepharose chromatography. The resulting A280-absorbing fractions that eluted at about 0.2 to 0.4M ammonium sulphate were pooled and regarded as Product B. Starting with a nominal 100mg of fully reduced SCR1-3/cys, Product B contained 17mg. The product contained one major species by non-reduced SDS PAGE with an estimated purity of >90% and an apparent molecular

Example 4 Expression and isolation of SCR1-3/switch fusion (SEQ ID NO 7)

H_2N-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH

(a) Construction of plasmid pDB1031 encoding SCR1-3/switch

Fragment 1 and fragment 2 of pDB1013-5 were the same in the as Example 3 above. Two oligonucleotides, SEQ ID No.3 and SEQ ID No. 4, prepared by Cruachem were annealed to give a final DNA concentration of 100 pmoles/ul. The annealed oligo has an *Ban*I/*Eco*RI overhang and duplicates the sequence at the 3' end of pDB1013-5 but in addition contains 17 additional codons coding for DGPKKKKKKSPSKSSGC just before the stop codon. This is fragment 4.

Fragments 1,2 and 4 were ligated with T4 DNA ligase in a single reaction to give pDB1031. The ligated plasmid was transfomed into competent *E. coli* JM109. Resulting colonies were analysed by restriction endonuclease digestion and DNA sequencing confirmed that the encoded amino acid sequence of SCR1-3 (SEQ ID 27 of WO 94/00571) had been altered by C terminal addition of amino acids DGPKKKKKKSPSKSSGC to give SEQ ID No. 7.

(b) Expression of SCR1-3/switch from pDB1031

pDB1031 was transformed into calcium chloride competent *E. coli* BL21(DE3) and resultant colonies were isolated and checked for plasmid content. To express protein from pDB1031 in *E. coli* BL21(DE3), a single colony was inoculated into 10 ml LB-phosphate media (20g/L tryptone, 15g/L yeast extract, 0.8g/L NaCl, 0.2g/L Na_2HPO_4, 0.1g/L KH_2PO_4) containing 50ug/ml ampicillin. The culture was grown for 6 hours at 37°C, 230 r.p.m. before being used to inoculate 100 ml of the same media containing 50 ug/ml ampicillin. Growth was under the same conditions overnight. 25 ml of each culture were then used to inoculate 600 ml of the same media with 50 ug/ml ampicillin in 3 L erlenmeyer flasks. Cells were grown to an OD of 0.8 - 1.0 at A_{600} nm. IPTG (isopropyl B-D galactopyranoside) was added to a final concentration of 1 mM and cells allowed to continue growth for a further 3 - 4 hours before harvesting by centrifugation at 8000 g/10 min. The cell pellet was frozen at -40 degrees C.

(c) Isolation, refolding, purification and formulation of SCR1-3/switch

The methods described are essentially those detailed in Dodd I. et al (1995) Protein Expression and Purification 6 727-736, with some modifications.

i) Isolation of solubilised inclusion bodies

The frozen cell pellet of *E. coli* BL21(DE3) (pDB1031) was thawed and resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM PMSF pH 8.0 at a ratio of 33 ml for each litre of culture pellet. The suspension was transferred to a glass beaker surrounded by ice and sonicated (Heat systems - Ultrasonics W380; 50 x 50% pulse, pulse time = 5 sec.) for typically 3 - 6 minutes. The disrupted pellet was then frozen and stored at -80°C. Approx. 1d later the sonicate was thawed and centrifuged at approx 8000g for 20 min. The pellet was resuspended in 20 mM Tris/8M urea/1 mM EDTA/50 mM 2-mercaptoethanol pH 8.5 (240ml) at room temperature by vigorous swirling, then left for 1h at room temperature followed by 5 days at 4° C.

ii) Preliminary purification using SP-Sepharose

To the viscous solution was added SP-Sepharose FF (approx. 30g wet weight) that had been water washed and suction dried. The mixture was swirled vigorously and left static for approx. 2h at room temperature. The supernatant was decanted, sampled and discarded. The remaining slurry was resuspended to a uniform suspension and poured into a glass jacket and allowed to settle into a packed bed. The column was equilibrated with 0.02M Tris/8M urea/0.05M 2-mercaptoethanol/0.001 M EDTA pH 8.5.at 4°C. When the A_{280} of the eluate had stabilised at baseline, the buffer was changed to equilibration buffer additionally containing 1M NaCl. A single A_{280} peak was eluted by the 1M NaCl-containing buffer; the volume was approx. 50ml. The protein concentration of the solution was estimated by A_{280} determination, using a molar extinction coefficient of 25000 cm^{-1},of a sample that had been buffer-exchanged (Sephadex G25) into 50 mM formic acid. This showed the product had a protein concentration of 2.8mg/ml. Analysis by SDS PAGE/stain showed a major band (approx 80%) at about 23 000Da. The solution was stored at -40°C.

iii) Folding and further processing

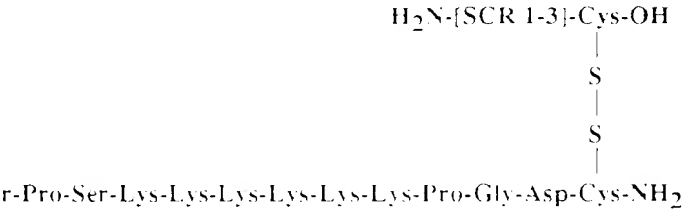
14ml of the SP-Sepharose-purified product was added gradually over a 1 min period to 430 ml freshly prepared, cold 0.05M Hepes/2 M sodium chloride/1mM EDTA pH 8.0 with continuous swirling, and left static for 1 h/4°C. Reduced glutathione (GSH) was added to 1 mM and oxidised glutathione (GSSG) was added to 0.5 mM. The solution was clear and was left static approx 2-3°C for 3 d. The solution was then ultrafiltered using a YM10 membrane to a final retentate volume of about 34 ml; the retentate was slightly cloudy. It was then spun at 25 000g for 15 mins and the supernatant buffer-exchanged into 0.1M sodium phosphate pH 7.0 (40 ml). This

(3.0ml) and stored at -40 degrees C. Absorbance measurements at 412nm on the solution prior to ultrafiltration suggested 25% derivatisation with DTNB.

(d) Alternative isolation, refolding, purification and formulation of SCR1-3/switch

The method was similar to that described in (c) above, except that following the ultrafiltration step after refolding it more closely followed the procedures described in Dodd et al (*op cit.*). Notably, the ultrafiltered retentate post refolding was immediately treated with ammonium sulphate followed by clarification by centrifugation and Butyl Sepharose chromatography. The resulting A280-absorbing fractions that eluted at about 0.2 to 0.4M ammonium sulphate were pooled and regarded as initial product. Additional treatment with TCEP essentially as above, followed by DTNB yielded a final product at 10uM final protein concentration. The final product contained one major species by non-reduced SDS PAGE with an estimated purity of >90% and an apparent molecular weight of 23 000 and contained about 2 moles TNB per mole of protein.

Example 5 Preparation of [SCR1-3]-Cys-S-S-[MSWP-1] (SEQ ID No. 8)



(a) Product A of Example 3(c) (1.5ml) was treated with dithiothreitol (30 ul of 0.5 M in water, final concentration 10mM) for 60 min at 4°C to give the free peptide SEQ ID NO 6. The yellow solution was gel filtered at 4°C on a small column of Sephadex G-25 (PD-10, Pharmacia) into 0.05M Hepes.HCl buffer pH 7.5 (3.0ml). The slightly cloudy solution was mixed with a solution of MSWP-1 (Example 2) (3.8mM dithiopyridyl equivalents, 150 ul) to a final concentration of 0.18mM (~8 molar equivalents). The mixture was held for 2h on ice and then gel filtered as before but using 2 PD10 columns (1.6 ml applied, 3.2ml eluted). The final eluate was not cloudy and was stored frozen at -70°C in aliquots of 0.4ml.

(b) [SCR1-3]-Cys protein product B described in Example 3(d) (1.5ml; 31uM protein) was mixed with TCEP (20mM; 0.007ml) and incubated at room temperature for 23 h to give the free peptide SEQ ID NO 6. MSWP-1 (Example 2) (10mM; 0.093ml) was added and the solution incubated for a further 4 h. (0.75ml of the final solution was

buffer-exchanged into 50mM formic acid and aliquots left in solution or lyophilised. The product was >80% pure by SDS PAGE and had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000. The lyophilisate was easily soluble in 50mM formic acid at an estimated protein concentration of 2 mg/ml.

(c) [SCR1-3]-Cys protein product B described in Example 3(d) (21.6ml; 31uM protein) was mixed with TCEP (20mM; 0.1ml) and incubated at room temperature for 22h to give the free peptide SEQ ID NO 6. MSWP-1 (20mM in 0.1M sodium phosphate pH 7.0; 0.67ml) was added and the solution incubated for a further 4 h. All 22ml was buffer-exchanged into 50mM formic acid using Sephadex G50 (Vt 160ml). Three A280 peaks were obtained. The first one, eluting at volume 56 -106ml, was the title compound according to SDS PAGE analysis. The fraction was aliquoted and aliquots stored at - 40 degrees C or lyophilised. Amino acid analysis of the prelyophilisation solution indicated a protein concentration of 0.42mg/ml. A280 (1cm path length) was 0.44. C8 reverse phase HPLC and SDS PAGE both indicated a purity of approx 80%. The latter technique showed the major band had an apparent molecular weight of 23 000, clearly shifted from the original parent molecular weight of 21 000; on reduction the 23 000 band shifted to two bands with molecular weights of approx 21 000 and approx 5 000. The lyophilisate was easily soluble in 50mM formic acid or in PBS 'A' (Dulbecco) at a protein concentration of 6mg/ml.

Example 6 Preparation of [SCR1-3/switch fusion]disulphide linked to [MAET]

H_2N-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH

$CH_3(CH_2)_{12}CONH-(CH_2)_2-S-S$

Title compound can be synthesised using TNB-activated SCR1-3/switch prepared as in Example 4(d). The TNB-activated SCR1-3/switch is mixed with a molar excess of MAET (Example 1), which might be typically made up at 2.0mg/ml in DMSO, equivalent to about 3mM free thiol. Typical reaction conditions would be 1 to 4 hours at room temperature or overnight at 4 degrees C using a protein concentration of 1 to 100 uM. The reaction may be monitored by checking the generation of yellow colour, which is caused by the release of free TNB ion. Once the reaction is complete the solution may be buffer exchanged into a suitable buffer, for example 0.1M sodium phosphate pH 7.0,

Example 7 Preparation of [SCR1-3/switch fusion] disulphide linked to [MSWP_1] (SEQ ID No 9)

H_2N-[SCR 1-3]-Asp-Gly-Pro-Lys-Lys-Lys-Lys-Lys-Lys-Ser-Pro-Ser-Lys-Ser-Ser-Gly-Cys-OH
|
S
|
S
|
N-(Myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

Method (a)

0.02ml of MSWP-1 (Example 2, 10mM in 0.1M sodium phosphate pH 7.0) was mixed with 0.005ml of TCEP (20mM in 50mM Hepes) and left for 10 min at room temperature. The resultant solution was Solution A containing the myristoylated peptide of SEQ ID NO 5. TNB-activated SCR1-3/switch prepared in a similar way to that described in Example 4(c) (0.3ml; 15uM in 0.1M sodium phosphate pH 7.0) was mixed with 0.0056ml of Solution A to give a theoretical MSWP-SH molar excess of five-fold over protein. The mixture was left for 4h at room temperature followed by 18h at 4 degrees C. Analysis by SDS PAGE followed by protein staining indicated one major band at apparent Mr 23K, corresponding to unreacted protein, and a minor band at apparent Mr 26K, corresponding to title protein.

Method (b)

TNB-activated SCR1-3/switch product (10uM; 0.43ml) prepared in a similar way to that described in Example 4(d) was mixed with TCEP (5mM; 0.0026ml) and incubated for 17h at room temperature to yield the free fusion protein SEQ ID NO 7. MSWP-1 (10mM; 0.0086ml) was added and incubation was continued for a further 4h. Small particles or crystals were present in the solution, but it was otherwise clear. The particulate solution was buffer-exchanged into 50mM formic acid (1.0ml), aliquoted and frozen. Analysis by SDS PAGE under non-reducing conditions showed a number of bands, which included a species with an apparent molecular weight of 25 000 - the target species.

Example 8 Preparation of [CR1-1929]-Cys-S-S-[MSWP-1] (SEQ ID NO 10)

H_2N-[CR1(1-1929)]-Cys-OH
|
S
|
S
|
N-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

Human complement receptor 1 (CR1, CD35) is a known regulator of complement activation which has been produced in a recombinant soluble form containing all of the extracellular SCR domains of a major natural allotype (Fearon et al, WO 89/09220, WO 91/05047). This form (sCR1) has been expressed as an active protein in Chinese Hamster Ovary (CHO) cells. Mutagenesis of the DNA sequence immediately downstream of the codon for Cys-1924 is performed to generate a new C-terminal cysteine residue.

A suitable example of a modified terminus of the cDNA sequence of sCR1 is as follows:

(5909) Bal I (5914)

.....CCT CTG GCC AAA TGT ACC TCT CGT GCA CAT TGC TGA

The codon Asp-1930 in CR1 is replaced by that for a Cysteine (followed by a stop codon to generate a soluble protein) through ligation of a modified oligonucleotide to the unique Bal I restriction endonuclease site at position 5914 (numbering from Fearon et al, 1989,1991).

Expression of this modified cDNA in CHO cells and isolation of the product by standard chromatographic procedures generates a modified sCR1 protein which can be treated as in Example 5 (a) or (b) to couple it to MSWP-1 (Example 2) to yield the title compound.

Biological Activity

Anti-complement Activity Measured by the Haemolysis of Sheep Erythrocytes

Functional activity of complement inhibitors was assessed by measuring the inhibition of complement mediated lysis of sheep erythrocytes sensitised with rabbit antibodies (Diamedix Corporation, Miami, USA). Human serum diluted 1:500 in 0.1 M Hepes/ 0.15 M NaCl pH 7.4 buffer was the source of complement and was prepared from a pool of volunteers essentially as described in (Dacie & Lewis, 1975). Briefly, blood was warmed to 37°C for 5 minutes, the clot removed and the remaining serum clarified by centrifugation. The serum fraction was split into small aliquots and stored at -196°C. Aliquots were thawed as required and diluted in the Hepes buffer immediately before use.

Inhibition of complement-mediated lysis of sensitised sheep erythrocytes was measured using a standard haemolytic assay using a v-bottom microtitre plate format as

of the 1/125 diluted serum for 15 minutes at 37°C . 100 µl of prewarmed sensitised sheep erythrocytes were added for 1 hour at 37°C in a final reaction volume of 200 µl . Samples were spun at 300g at 4°C for 15 minutes before transferring 150 µl of supernatant to flat bottom microtitre plates and determining the absorption at 410 nm, which reflects the amount of lysis in each test solution. Maximum lysis was determined by incubating serum with erythrocytes in the absence of any inhibitor (E+S) from which the proportion of background lysis had been subtracted (determined by incubating erythrocytes with buffer (E). The background lysis by inhibitor was assessed by incubating inhibitor with erythrocytes (E+I) and then subtracting that from test samples (E+I+S). Inhibition was expressed as a fraction of the total cell lysis such that IH50 represents the concentration of inhibitor required to give 50% inhibition of lysis.

Maximum Lysis: A max = (E+S) - (E)

Lysis in presence of inhibitor: Ao = (E+I+S) - (E+I)

Amount of inhibition: $IH = \frac{Amax-Ao}{Amax}$

Results

Compound	IH50
WO94/00571 SEQ ID NO 27	0.2 - 0.3 ug/ml
Example 3*	0.65 ug/ml (mean of two)
Example 4*	0.3-1.0 ug/ml (n = 3)
Example 5a Example 5b Example 5c	0.014 ug/ml <0.001 ug/ml 0.001 ug/ml
Example 7a Example 7b	0.02 ug/ml ~0.01 ug/ml

* As 2-mercaptoethanol/glutathione derivatives

The above data show that:

1. The complement inhibitory activities of the 'base' protein (SCR1-3 of human complement receptor 1 of WO94/00571) and its derivatives with either an additional C-terminal cysteine (SCR1-3/cys, Example 3) or a single cationic 'switch' sequence (SCR1-3/switch, Example 4) are very similar.

2. However, incorporation of two membrane binding elements (electrostatic switch and myristoyl) by addition of MSWP-1 (which contains both elements) to SCR1-3/cys or three membrane binding elements by addition of the MSWP-1 to the SCR1-3/switch construct results in products which are significantly more potent (~20-200x) than the base or single membrane binding element proteins. This increase in potency in an assay which depends on a membrane event (cytolysis by the membrane attack complex of complement) can be attributed to membrane targeting of the cytolysis inhibitor proteins by the combination of two membrane binding elements.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCACCGCAGTGCATCATCCCGAACAAATGCTAATAAA

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCTTTTATTAGCATTTGTTCGGGATGATGCACTGCG

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCACCGCAGTGCATCATCCCGAACAAAGACGGTCCGAAAAAGAAGAAAAAG
AAATCTCCGTCCAAATCTTCCGGTTGCTAATAAA

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTTTTATTAGCAACCGGAAGATTTGGACGGAGATTTCTTTTTCTTCTTTTTC
GGACCGTCTTTGTTCGGGATGATGCACTGCG

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-NH_2

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 198 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Gln	Cys	Asn	Ala	Pro	Glu	Trp	Leu	Pro	Phe	Ala	Arg	Pro	Thr	Asn
1				5				10					15		

Leu	Thr	Asp	Glu	Phe	Glu	Phe	Pro	Ile	Gly	Thr	Tyr	Leu	Asn	Tyr	Glu
			20				25					30		

Cys	Arg	Pro	Gly	Tyr	Ser	Gly	Arg	Pro	Phe	Ser	Ile	Ile	Cys	Leu	Lys
		35					40					45		

Asn	Ser	Val	Trp	Thr	Gly	Ala	Lys	Asp	Arg	Cys	Arg	Arg	Lys	Ser	Cys
	50					55					60				

Arg	Asn	Pro	Pro	Asp	Pro	Val	Asn	Gly	Met	Val	His	Val	Ile	Lys	Gly
65					70					75					80

Ile Gln Leu Gly Ser Gln Ile Lys Tyr Ser Tyr Thr Lys Gly Tyr Arg
85 90 95

Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
100 105 110

Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
115 120 125

Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
130 135 140

Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
145 150 155 160

Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
165 170 175

Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
180 185 190

Ile Ile Pro Asn Lys Cys
195

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 217 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
1 5 10 15

Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
20 25 30

Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
35 40 45

Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
50 55 60

Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
65 70 75 80

Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
85 90 95

Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
100 105 110

Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
115 120 125

Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
130 135 140

Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
145 150 155 160

Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
165 170 175

Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
180 185 190

Ile Ile Pro Asn Lys Asp Gly Pro Lys Lys Lys Lys Lys Lys Ser Pro

Ser Lys Ser Ser Gly Cys

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 215 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear, 2 polypeptide chains disulphide linked

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
1 5 10 15

Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
20 25 30

Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
35 40 45

Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
50 55

Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
65 70 75 80

Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg

Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
100 105 110

Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
115 120 125

Pro Pro Thr Ile Thr Asn Gly Asp Phe Ile Ser Thr Asn Arg Glu Asn
130 135 140

Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
145 150 155 160

Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr
165 170 175

Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Pro Ala Pro Gln Cys
180 185 190

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Ile Ile Pro Asn Lys Cys-S-S-Cys Asp Gly Pro Lys Lys Lys Lys Lys

CO_2H $CONH_2$

Lys Ser Pro Ser Lys Ser Ser Gly (N-Myristoyl)

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear, 1 polypeptide chains disulphide linked

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Gln Cys Asn Ala Pro Glu Trp Leu Pro Phe Ala Arg Pro Thr Asn
1 5 10 15

Leu Thr Asp Glu Phe Glu Phe Pro Ile Gly Thr Tyr Leu Asn Tyr Glu
20 25 30

Cys Arg Pro Gly Tyr Ser Gly Arg Pro Phe Ser Ile Ile Cys Leu Lys
35 40 45

Asn Ser Val Trp Thr Gly Ala Lys Asp Arg Cys Arg Arg Lys Ser Cys
50 55 60

Arg Asn Pro Pro Asp Pro Val Asn Gly Met Val His Val Ile Lys Gly
65 70 75 80

Ile Gln Phe Gly Ser Gln Ile Lys Tyr Ser Cys Thr Lys Gly Tyr Arg
85 90 95

Leu Ile Gly Ser Ser Ser Ala Thr Cys Ile Ile Ser Gly Asp Thr Val
100 105 110

Ile Trp Asp Asn Glu Thr Pro Ile Cys Asp Arg Ile Pro Cys Gly Leu
115 120 125

Pro Pro Thr Ile Thr Asn Gly Arg Phe Ile Ser Thr Asn Arg Glu Asn
130 135 140

Phe His Tyr Gly Ser Val Val Thr Tyr Arg Cys Asn Pro Gly Ser Gly
145 150 155 160

Gly Arg Lys Val Phe Glu Leu Val Gly Glu Pro Ser Ile Tyr Cys Thr

Ser Asn Asp Asp Gln Val Gly Ile Trp Ser Gly Tr Ala Pro Gln Cys
180 185 190

Ile Ile Pro Asn Lys Asp Gly Pro Lys Lys Lys Lys Lys Lys Ser Pro

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Ser Lys Ser Ser Gly Cys-S-S-Cys Asp Gly Pro Lys Lys Lys Lys Lys

CO2H CONH2

Lys Ser Pro Ser Lys Ser Ser Gly (N-Myristoyl)

SEQ ID NO 10

1 QCNAPEWLPF ARPTNLTDEF EPPIGTYLNY ECRPGYSGRP

41 FSIICLKNSV WTGAKDRCRR KSCRNPPDPV NGMVHVIKGI

81 QFGSQIKYSC TKGYRLIGSS SATCIISGDT VIWDNETPIC

121 DRIPCGLPPT ITNGDFISTN RENFHYGSVV TYRCNPGSGG

161 RKVFELVGEP SIYCTSNDDQ VGIWSGPAPQ CIIPNKCTPP

201 NVENGILVSD NRSLFSLNEV VEFRCQPGFV MKGPRRVKCQ

241 ALNKWEPELP SCSRVCQPPP DVLHAERTQR DKDNFSPGQE

281 VFYSCEPGYD LRGAASMRCT PQGDWSPAAP TCEVKSCDDF

321 MGQLLNGRVL FPVNLQLGAK VDFVCDEGFQ LKGSSASYCV

361 LAGMESLWNS SVPVCEQIFC PSPPVIPNGR HTGKPLEVFR

401 FGKAVNYTCD PHPDRGTSFD LIGESTIRCT SDPQGNGVWS

441 SPAPRCGILG HCQAPDHFLF AKLKTQTNAS DFPIGTSLKY

481 ECRPEYYGRP FSITCLDNLV WSSPKDVCKR KSCKTPPDPV

521 NGMVHVITDI QVGSRINYSC TTGHRLIGHS SAECILSGNA

561 AHWSTKPPIC QRIPCGLPPT IANGDFISTN RENFHYGSVV

601 TYRCNPGSGG RKVFELVGEP SIYCTSNDDQ VGIWSGPAPQ

641 CIIPNKCTPP NVENGILVSD NRSLFSLNEV VEFRCQPGFV

681 MKGPRRVKCQ ALNKWEPELP SCSRVCQPPP DVLHAERTQR

721 DKDNFSPGQE VFYSCEPGYD LRGAASMRCT PQGDWSPAAP

761 TCEVKSCDDF MGQLLNGRVL FPVNLQLGAK VDFVCDEGFQ

801 LKGSSASYCV LAGMESLWNS SVPVCEQIFC PSPPVIPNGR

841 HTGKPLEVFP FGKAVNYTCD PHPDRGTSFD LIGESTIRCT

881 SDPQGNGVWS SPAPRCGILG HCQAPDHFLF AKLKTQTNAS

921 DFPIGTSLKY ECRPEYYGRP FSITCLDNLV WSSPKDVCKR

961 KSCKTPPDPV NGMVHVITDI QVGSRINYSC TTGHRLIGHS

1001 SAECILSGNT AHWSTKPPIC QRIPCGLPPT IANGDFISTN

1041 RENFHYGSVV TYRCNLGSRG RKVFELVGEP SIYCTSNDDQ

1081 VGIWSGPAPQ CIIPNKCTPP NVENGILVSD NRSLFSLNEV

1121 VEFRCQPGFV MKGPRRVKCQ ALNKWEPELP SCSRVCQPPP

1241 VSFVCDEGFR LKGSSVSHCV LVGMRSLWNN SVPVCEHIFC

1281 PNPPAILNGR HTGTPSGDIP YGKEISYTCD PHPDRGMTFN

1321 LIGESTIRCT SDPHGNGVWS SPAPRCELSV RAGHCKTPEQ

1361 FPFASPTIPI NDFEFPVGTS LNYECRPGYF GKMFSISCLE

1401 NLVWSSVEDN CRRKSCGPPP EPFNGMVHIN TDTQFGSTVN

1441 YSCNEGFRLI GSPSTTCLVS GNNVTWDKKA PICEIISCEP

1481 PPTISNGDFY SNNRTSFHNG TVVTYQCHTG PDGEQLFELV

1521 GERSIYCTSK DDQVGVWSSP PPRCISTNKC TAPEVENAIR

1561 VPGNRSFFSL TEIIRFRCQP GFVMVGSHTV QCQTNGRWGP

1601 KLPHCSRVCQ PPPEILHGEH TLSHQDNFSP GQEVFYSCEP

1641 SYDLFGAASL HCTPQGDWSP EAPRCTVNSC DDFLGQLPHG

1681 RVLLPLNLQL GAKVSFVCDE GFRLKGRSAS HCVLAGMKAL

1721 WNSSVPVCEQ IFCPNPPAIL NGRHTGTPFG DIPYGKEISY

1761 ACDTHPDRGM TFNLIGESSI RCTSDPQGNG VWSSPAPRCE

1801 LSVPAACPHP PKIQNGHYIG GHVSLYLPGM TISYTCDPGY

1841 LLVGKGFIFC TDQGIWSQLD HYCKEVNCSF PLPMNGISKE

1881 LEMKKVYHYG DYVTLKCEDG YTLEGSPWSQ CQADDRWDPP

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                 ->        <-
1921 LAKCTSRAHC]-S-S-[CDGPKKHKKKSPSKSSG]-(N-Myristoyl)
                  |         |
                  CO2H      CONH2
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In SEQ ID NO 10, peptide sequences are given in square brackets in single letter amino acid code.